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Enhancing Osteoclastic Resorption for the Prevention and Treatment of Heterotopic Ossification

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14. ABSTRACT

Bone-resorbing osteoclasts normally resorb ectopic mineral in their innate immune role. Therefore, we hypothesize that: blockade of osteoclastic bone resorption is required for heterotopic bone formation and that ***lifting repression will allow resorption*** of ectopic bone in heterotopic ossification.

Purpose: to test methods to enhance osteoclast activity to reduce HO.

Scope: This work will use a mouse model of HO to test two different mechanisms to enhance osteoclast formation and function. The first is treatment of HO mice with exogenous RANKL, the key osteoclast formation cytokine. A second approach is by antibody inhibition of OPG, the natural antagonist of RANKL. Results from the second approach will be tested in OPG knockout mice.

Major findings: We have established colonies of all mice required for the experiments. We have established the mouse model of HO and characterized it by histological and histochemical analysis, X-ray, and micro-CT imaging. In addition, using osteoclast cell culture, we have screened and identified the best candidate anti-OPG antibody for inhibition experiments. We ARE testing anti-OPG antibody treatment in the HO model.

15. SUBJECT TERMS
Heterotopic ossification, Rehabilitation, Neuromusculoskeletal Injuries, Blast trauma, orthopaedic

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Introduction:

The purpose of this study is to test the hypothesis that osteoclastic bone resorption is inhibited in heterotopic ossification and that if inhibition is lifted, heterotopic ossification will be lessened or reversed. The proposal consists primarily of three main experiments, addressing two different mechanisms to induce osteoclastic resorption of heterotopic bone. Using a mouse model of heterotopic ossification we will test activation of osteoclasts by treatment with exogenous RANKL, blockade of the RANKL decoy receptor OPG with antibodies, and testing the ability of OPG knockout mice to develop heterotopic ossification.

Body:

Overall we have made significant progress to date despite challenges. Regulatory review and approval of animal experiments has been completed at the new institution, the University of Florida (UF) (as required for Tasks 1 and 2). Army ACURO review and approval of the new animal protocol has been completed (as required for Tasks 1 and 2). Biosafety approval is required in addition and has been received. The caACVR1 mouse line has been transferred to UF and has passed quarantine. The caACVR1 mice have been bred to homozygosity for the transgene and the colony is undergoing expansion. Several animals are currently on protocol and in vivo experiments have begun at UF (see Tasks 6). The caACVR1 transgene is being bred into the OPG knockout background at UF. We have identified OPG -/+ animals carrying the caACVR1 transgene and are currently crossing to produce the OPG-/- animals which we will test for homozygosity of the caACVR1 transgene by backcross to wild type C57 mice (Task 2). We have tested anti-OPG antibodies by in vitro osteoclast formation assays and have identified the best candidates for blockade in vivo (Task 5). We have tested our adeno-GFP in wild type mice and see GFP expression. We have tested our adeno-Cre preparations in caACVR1 mice and have seen GFP expression in injected mice, indicating expression of the Cre recombinase in vivo (testing for Task 3). We have demonstrated heterotopic bone formation in caACVR1 mice following injection of adeno-Cre and have documented the absence of heterotopic ossification with adeno-GFP injection. Heterotopic bone was demonstrated both histologically and by micro-computed tomography (micro-CT). Initial testing of anti-OPG antibody in the caACVR1 mouse model of HO was performed providing promising but preliminary results. HO has been induced in eighteen animals and half have been treated weekly with anti-OPG (IP injection) with the remainder receiving control goat IgG. Results are forthcoming. New litters of caAVCR1 animals continue to be born and will be enrolled in the protocol when they are 10 days of age. 6 pairs of caAVCR1 mice are now breeding on protocol which will provide us with a large number of animals for ongoing experiments, approximately 1 to 2 litters per week for use in Tasks 4 and 6.

Task 1. Breed sufficient caACVR1 (aka caAKL2) conditional transgenic mice and controls for *in vivo* experiments.
Months (1 – 24): Regulatory Review and Approval and breeding mice
Progress: Regulatory review completed
Our move to a new institution necessitated new regulatory review and approval of animal studies. We have received Biosafety approval for our animal experiments. The new animal protocol was approved by the local IACUC. In this instance, caACVR1 animals with HO were considered USDA Classification "E", which includes experiments in which pain or distress is not relieved with the use of anesthetics or analgesics. This was the judgment of the local IACUC. These animals were considered category "C" at the previous institution, with animals experiencing brief pain or distress. Analgesics cannot be employed since the HO bone formation is inherently an inflammatory process and blockade of inflammation could directly affect the model. This complication resulted in longer regulatory review by the local IACUC. Upon review and approval from the local IACUC, the new protocol was rapidly approved by the USAMRMC ACURO.

Months (4-24): Breeding ACVR1 animals
Progress: Ongoing
By the end of the reporting period we have 6 pairs of animals breeding. Each was shown to be homozygous for the caACVR1 transgene, rather than hemizygous (heterozygous) via back-cross to wildtype. Breeding to homozygosity was undertaken since hemizygous animals only produced HO in 2/3 of animals, as shown in **Figure 5** of Y1 yearly report and as noted for ***Task 6*** below.

Task 2. Breed sufficient OPG-/- mice and controls and cross in the caACVR1 transgene for *in vivo* experiments.
Months (6 – 30): Regulatory Review and Approval and breeding mice.
Progress: Regulatory review completed.
Please see progress for *Task 1* (above) for details of regulatory review and approval since OPG-/- animal experiments are conducted under the same IACUC approval and USAMRMC ACURO approval. OPG-/- animals originally bred poorly since they often consumed their offspring. This may have been either environmental or the effect of young parents since we have not seen much of this recently. We have crossed caACVR1 transgenic mice X OPG -/- and have identified OPG-/+; caACVR1 transgenic animals in the first generation. Nine pairs of these animals are currently being crossed and we expect ¼ of the offspring to be OPG-/- and ¾ of those to carry the transgene. The first litters from most pairs are born and will be screened once weaned. We anticipate that several OPG-/-; caACVR1 transgenic mice will be identified in the first generation. OPG-/-; caACVR1 transgenic mice will be backcrossed to C57Bl/6 wild type to identify those homozygous for the transgene (approximately 1 in 3 of the OPG-/- ;caACVR1 positive animals. This screening will take approximately 2 months and the colony will be expanded thereafter.

Task 3. Produce sufficient adenoviral-Cre (ad-Cre) and control virus (ad-GFP) at high titer for induction of HO in the mouse model.
Months (1 – 30)
Progress: Ongoing
Ad-Cre adenovirus was tested *in vivo* and now routinely produces strong HO in the homozygous caACVR1 animals (see ***Task 6*** below, and **Figure 1**).

Task 4. Test RANKL effects on heterotopic ossification development.
Months (6 – 24).
Enrolment and injection of animals will proceed as they become available. ***Task 6*** was begun prior to ***Task 4*** due to the potentially positive preliminary results demonstrated using the anti-OPG approach and in response to the rapid completion of ***Task 5***.

Task 5. Test anti-OPG antibodies for function blocking in the mouse model in vitro.
Months (3 – 12)
Progress: Completed
Work on Task 5 was completed in Year 1 as we have tested and identified 2 commercially available goat anti-OPG polyclonal antibody preparations that show function blocking of OPG in culture of RAW264.7 cells with RANKL (R&D Systems cat# AF459, and Sigma cat# O1139). Both commercial preps function similarly as the dose required to reverse OPG blockade of osteoclastogenesis is essentially the same (see **Figure 1** and **Figure 2** in Year 1 Report).

Task 6. Test anti-OPG effects on heterotopic ossification development (using mice produced in ***Task 1*** and ad-Cre from ***Task 3***)
Months (12 - 24)
Progress: In year 1 we demonstrated HO production in caACVR1 animals following ad-Cre injection vs ad-GFP injection. Initial experiments were performed at BIDMC in Boston and under the local IACUC and USAMRMC ACURO approved protocol prior to the laboratory move. Analysis of these results was completed at the University of Florida. Plain radiographs of caACVR1 animals injected with ad-Cre and ad-GFP show radiographically evident heterotopic bone formation in the Ad-Cre injected animal (see **Figure 3** in Year 1 Report). We have demonstrated heterotopic bone formation as well as the presence of cartilage histologically (see **Figure 4** in Year 1 Report). We performed an initial test of anti-OPG treatment of HO by injecting 6 caACVR1 animals with ad-Cre and treated ½ of them with anti-OPG antibody. Animals were sacrificed after 10 days for micro-CT and histology. Micro-CT imaging of hind quarters shows HO formation in 2 of 3 controls and little in 1 of 3 anti-OPG treated animals (see **Figure 5**, Year 1 Report). While these results provided preliminary evidence supporting our hypothesis and indicate that our approach may work, they also indicated variable HO production and perhaps very low HO induction in a proportion of the ad-Cre injected caACVR1 animals. We hypothesized that these were animals with only one copy of the transgene from the cross of parents heterozygous (hemizygous) for the transgene. In order to identify mice homozygous for the transgene, in Year 2 we backcrossed to wild type C57Bl/6 and screened offspring for caACVR1. Mice producing all transgenic offspring are themselves homozygous and several founders were identified. Strong HO was induced in each of the first 17 caACVR1 homozygous animals 30 days following injection with ad-Cre. Micro-Ct analysis of HO was performed on five male and six female animals as shown in **Figure 1**. Robust HO formation is shown in all imaged animals regardless of sex. HO is restricted to the injected side with the contralateral side remaining uninvolved.
In addition to providing a more robust animal model, breeding homozygotes will simplify breeding since we no longer need to genotype animals. The homozygous caACVR1 colony has been expanded with at least 6 pairs producing experimental animals and an additional 4 pairs currently producing replacement breeders. We now can anticipate two litters per week of 6 to 12 pups, which will allow us to continuously enroll animals in the protocol and to replace aged breeders.

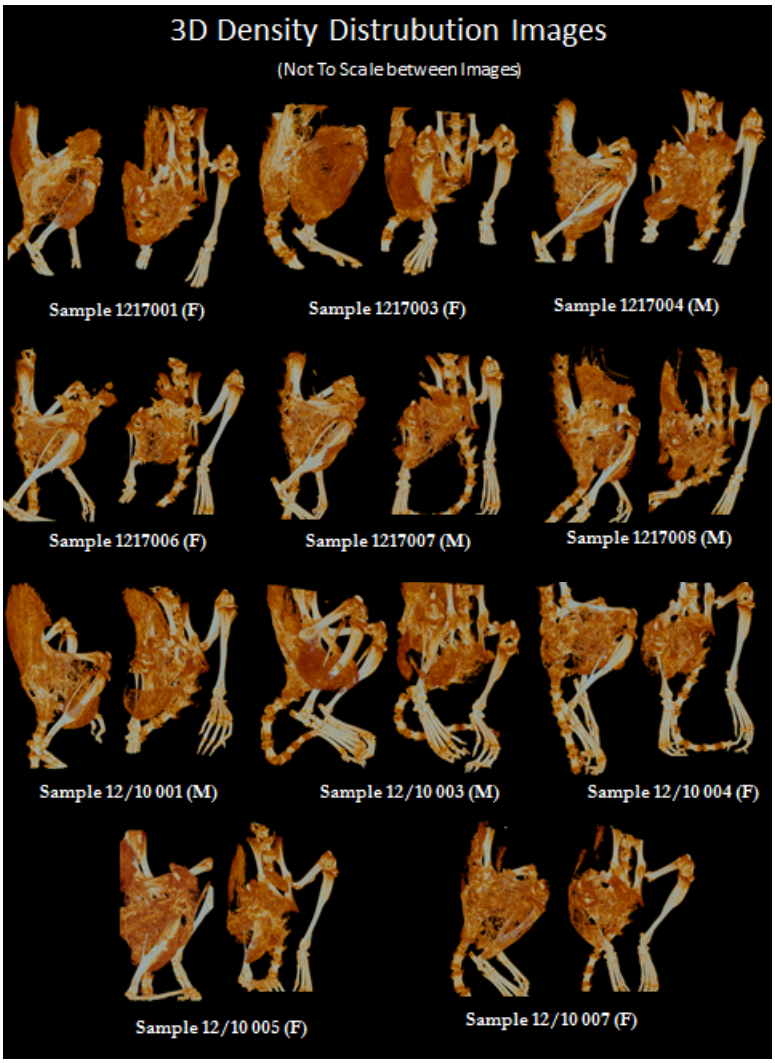

Figure 1. Micro –Ct images of HO induced in 5 male (M) and 6 female (F) caACVR1 homozygous animals. There are two images per specimen: (1) lateral view of HO affected side and, (2) near frontal view of specimen, showing contralateral limb unaffected by HO. HO was induced with 20 ul of an adeno-Cre prep with an apparent titer of 4.8 X 10^{10} pfu/ml (9.6 X 10^{8} pfu in 20ul). HO development is extensive in all specimens and independent of the sex of the animal. HO often fuses with the pelvis and lower spine. There appears to be no clear differences between specimens. These results indicate that we can produce robust HO in all injected animals and we can use male or female animals. We anticipate a need to titrate the administered adeno-Cre to produce a more localized HO reaction for treatment experiments. In addition these results provide a data set for determination of how best to quantify HO. For example, HO could be quantified as total ectopic mineral per animal or a region of interest (ROI) could be determined in the injected limb and normalized to the same ROI on the contralateral limb. The latter approach would take into account any systemic bone loss due to treatment with anti-OPG.

Task 7. Test HO development in OPG-/- mouse background (using mice produced in ***Task 1*** and ***Task 2***, with ad-Cre from ***Task 3***).
Breeding is underway (***Task 2***) to produce experimental animals. Enrolment and injection will proceed as animals become available in Year 3.

Key Research Accomplishments:

- No results from completed experiments are available for the reporting period.

Reportable Outcomes:

Seminar Presentations:

1. McHugh, Kevin, P. “Osteoclast Differentiation in Peri-implant Osteolysis and the Role of Osteoclasts in Heterotopic Ossification.”
   Lecture, Division of Orthopaedic Research,
   Rhode Island Hospital & Brown Medical School,
   Providence, RI, September 24, 2011.

2. McHugh, Kevin, P. “Osteoclasts, Osteoblasts & Heterotopic Ossification.”
   Lecture, Translational Research Seminar,
   College of Dentistry, University of Florida,
   Gainesville, FL, October 25, 2011.

3. McHugh, Kevin P. “An Innate Immune Role for Osteoclasts in Heterotopic Ossification.”
   Lecture, Research in Progress Seminar,
   Division of Rheumatology and Clinical Immunology, Department of Oral Biology, and Department of Pathology, Immunology, and Laboratory Medicine,
   College of Medicine, University of Florida,
   Gainesville, FL, November 30, 2011.

4. McHugh, Kevin P. “Regulation of Osteoclast Formation & Function by Bone Matrix.”
   Lecture, Cytoskeletal Research Group,
   Department of Cell Biology, University of Florida,
   Gainesville, FL, September 13, 2012.

Conclusions:

Significant progress has been made in the face of a very disruptive move. All reagents required have been procured and tested. Mouse colonies have been established and animal models are now in place and working properly. Laboratory personnel have been hired in the new lab. New personnel have been trained in the required procedures and are now able to genotype mice, perform IM and IP injections in 10 day old animals, perform range of motion assays on animals enrolled in the experimental protocol and induce strong and reproducible induce HO in the caACVR1 model.

We currently have several animals on protocol and are producing new subjects at a rapid pace. We anticipate that we will quickly recover from delays and will be able to successfully complete all experiments and tasks as described in the Statement of Work.

Our preliminary test of OPG blockade with anti-OPG antibody, and subsequent ongoing experiments indicate that our approach of enhancing osteoclastic bone resorption to decrease heterotopic bone formation is producing positive results. We anticipate that our results may be directly and quickly applied in a clinical / translational study, and a proposal with that goal is in preparation.

References:

None

Appendices:

None